10/535334
YC1/0605/00261

PA 1148521

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

March 30, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: *60/427,148*
FILING DATE: *November 18, 2002*

SG/03/261

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

N. WOODSON
Certifying Officer

11-19-2004 ... PRO

PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 C.F.R. § 1.53(b)(2).

Attorney Docket Number: 3165.01US01

INVENTOR(S) / APPLICANT(S)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (City and Either State or Foreign Country)
KWAN	Meng Hui		Singapore
ZHANG	Chunyan		Singapore
SAW	Lin Kiat		Singapore
TING	Dor Ngi		Singapore
POLLA	Dennis L.		Singapore (U.S. Citizen)

TITLE OF INVENTION (280 characters max)

MEMS-COMPATIBLE ENZYME DIGESTION METHOD FOR NUCLEIC ACID EXTRACTION FROM SOLID TISSUE

CORRESPONDENCE ADDRESS

Customer No. 24113
Curtis B. Herbert
Patterson, Thuente, Skaar & Christensen, P.A.
4800 IDS Center, 80 South 8th Street
Minneapolis, Minnesota 55402-2100 USA

ENCLOSED APPLICATION PARTS (check all that apply)

[X] Specification Number of Pages: 18
[X] Drawings Number of Sheets: 5
[ ] Applicant(s) is/are entitled to small entity status in accordance with 37 CFR 1.27.
[ ] CD-ROM or CD-R in duplicate, and Compact Disc Transmittal
[ ] ______________________

METHOD OF PAYMENT

[X] A check in the amount of $160.00 is enclosed to cover the provisional application filing fee. The Commissioner is hereby authorized to charge any additional filing fees and/or to credit any overpayment to our Deposit Account Number 16-0631.

The invention was made by an agency of the U.S. Government or under a contract with an agency of the U.S. Government.

[X] No. [ ] Yes. The name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

Curtis B. Herbert
Curtis B. Herbert
Registration. No. 45,443

Date: November 18, 2002

PROVISIONAL APPLICATION FILING ONLY

CERTIFICATE OF EXPRESS MAIL

"Express Mail" mailing label number: EL671576146US. Date of Deposit: November 18, 2002. I hereby certify that this paper is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Jeanne Truman
Name of Person Making Deposit

Jeanne Truman
Signature

MICROMECHANICAL-COMPATIBLE SYSTEMS AND METHODS FOR ENZYME DIGESTION OF SOLID TISSUE FOR NUCLEIC ACID EXTRACTION

BACKGROUND

Analysis of the nucleic acids in tissues is performed for many purposes, including forensic sciences, the study of diseases, and medical sciences. This study of the nucleic acids typically requires extracting the nucleic acids from the tissue. A step in nucleic acid extraction is tissue homogenization.

A tissue usually contains many cells that are joined together by a biological matrix that provides mechanical strength to the tissue. The tissue homogenization step breaks up the biological matrix. The biological matrix typically is rich in collagen, often as much as 90% collagen.

After the homogenization step, the cells must also be broken up in a cell disruption step so that the nucleic acids that they contain may be analyzed. The homogenization and cell disruption steps are typically accomplished simultaneously or the homogenization step breaks up some of the cells and the cell disruption step completes the cell disruption process. Figure 1 provides a flow chart of nucleic acid extraction and analysis, see also Huang et al., *Anal. Bioanal. Chem.*, 372, 49-65.

The tissue homogenization step conventionally involves using mechanical force to disrupt the tissue, and the cell disruption step conventionally involves using chemicals or enzymes. The mechanical tissue homogenization breaks up the tissue so that the chemicals or enzymes can penetrate the sample and the cells in the tissue. Without tissue homogenization, the chemicals or enzymes in the cell disruption step would only affect some of the cells in the

tissue sample. Tissue homogenization breaks up some of the cells, but the enzymatic and chemical treatments are needed to disrupt all of the cells and to help separate the nucleic acids from the rest of the cell. Other complex tasks to complete the analysis are performed after the nucleic acids have been extracted, including amplification and detection of the nucleic acids.

The task of preparing nucleic acids for analysis has conventionally been a time-consuming and labor-intensive process. Recent advances in μ-fluidics and microelectromechanical systems (MEMS) technology have led to the miniaturisation of many micro-scale analytical instruments. The advantages of miniaturisation in fluid processing include improved efficiency with regard to sample size, response times, cost, analytical performance, process control, integration, throughput and automation (de Mello, *Anal. Bioanal. Chem.* 372:12-13, 2002). The homogenization and cell disruption portions of the process, however, continue to be performed in a time-consuming and labor-intensive manner. Indeed, it has been difficult to automate, make robots, or make micromechanical devices that perform homogenization and cell disruption.

SUMMARY OF THE INVENTION

Embodiments of the invention are a device and a method of tissue homogenization that require no mechanical homogenization step so that automatic, robotic, or micromechanical approaches to tissue homogenization may be accomplished. Embodiments of the invention are a device and a method of homogenizing tissue using chemical enzymatic digestion, including proteolytic degradation. An embodiment of the invention is a device for tissue sample preparation that includes a micromechanical proteolytic tissue homogenization chamber. An embodiment of the invention is a system for tissue sample preparation that includes a micromechanical proteolytic tissue homogenization chamber and may include a

protease. Collagenase is a preferred protease since tissue are recognized to contain large amounts of collagen.

In some embodiments of the invention, the proteolytic tissue homogenization chamber may be operably connected to at least one other chamber. In various embodiments, the other chamber(s) are be used for holding a protease, holding buffers, holding protease inhibitors, holding stains or visualization agents, or serving a receptacles for waste products or nucleic acids. In various embodiments, the chambers are sized to have a volume of less than 100 μl, less than 50 μl, less than 10 μl, or less than 5 μl.

An embodiment of the invention is a method of using a device for homogenizing tissue using proteolytic degradation. An embodiment of the invention is a method of using a micromechanical proteolytic tissue homogenization chamber. An embodiment of the invention is a method of using a system for tissue sample preparation that includes a micromechanical proteolytic tissue homogenization chamber and may include a protease.

An embodiment of the invention is a method of preparing a tissue sample, the method comprising providing a tissue sample of less than about 10 mm^3 or less than about 3 mm^3 and exposing the tissue sample to a protease until the tissue is homogenized.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a flow chart of a conventional scheme for nucleic acid analysis;

Figure 2 depicts an agarose gel showing that some embodiments of the invention (lanes 3-6) are as effective as conventional methods (lane 7);

Figure 3A is a plan view of a microfluidic tissue digester incorporating a proteolytic tissue homogenization chamber;

Figure 3B is a perspective view of the device of Figure 3A;

Figure 4A is a side elevated view of a microfluidic tissue digester incorporating a proteolytic tissue homogenization chamber;

Figure 4B is a side elevated view of a microfluidic tissue digester incorporating a proteolytic tissue homogenization chamber;

Figure 4C is a perspective photograph of the microfluidic tissue digester of Figure 4A; and

Figure 5A depicts a plan for making a microfluidic tissue digester.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention provides methods and articles for preparing tissue for nucleic acid extraction that are adaptable for use with micromechanical devices and automated processes. An embodiment of the invention is a method for performing tissue homogenization without a step of mechanically disrupting the tissue. Instead, the tissue is disrupted using a protease. A protease can be applied as a solution that contacts the tissue and homogenizes it.

The process of adding a solution to a tissue can be performed quickly and requires no complex parts. This is advantageous because tissue homogenization may thereby be automated and may be incorporated into micromechanical devices. Micromechanical devices include biological microelectromechanical systems (bioMEMS) and fully automated complete micro total analytical systems (μTAS).

Further, the homogenization process may be performed so that the cells in the tissue are substantially not disrupted until the cell disruption step. After homogenization of the tissue, the cells of interest may thus advantageously be separated from the rest of the tissue so

that the contents of a desired subset of cells may be probed instead of all the cells in the tissue.

Further, since the cells may be kept intact through the tissue homogenization step, the RNAases in the cells are kept essentially within the lysosomes in the cells and are thereby sequestered within the cell. RNAses are proteases that destroy RNA polynucleic acids so that nucleic acid analysis is ineffective. RNAses are conventionally inhibited using RNAase inhibitors. Since RNAases may be essentially sequestered with the cells using an embodiment of the invention, the need for RNAase inhibitors, and the need for vigilance in their administration, may be eliminated. The intact cells are not necessarily viable. Intact refers to a state of the membrane of the cells, including the cellular wall and lysosomes. Viability refers to the ability to remain alive. Cells may thus be intact but unviable.

Avoiding the effects of RNAases is important. It is well known that RNAs are fragile and rapidly degradable by RNAses present in a tissue sample as well as contaminations from human sweat, including that present on fingertips. Other than ensuring that all instruments, containers, and working areas are RNAase-free, the technicians must be careful not to allow freshly harvested samples to remain at room temperature unpreserved, frozen samples to defrost, or mechanical tissue disruption to take place without the presence of nuclease inhibitors. Certain embodiments of the invention remove all these meddlesome technicalities. For example, a chamber of the bioMEMs can receive the sample immediately after biopsy or tissue harvest, potentially removing the need for preservation procedures. And a fully automated sample preparation requires no human interference greatly minimising contaminative nucleases found in human sweat.

Some conventional methods for isolating cells use proteases to treat a tissue. A protease is an enzyme that cleaves or catalyzes the cleavage of peptidic chemical bonds. A peptidic chemical bond is a chemical bond that joins two or more amino acids, for example: a bond formed between two amino acids of a protein. For example, Dwulet et al., in U.S. patent No. 5,952,215 and Uchida, in U.S. Patent No. 6,238,922, describe exposing tissue to the protease collagen. And Freshney describes exposing tissue to trypsin, see RI Freshney, Freshney's Culture of Animal Cells, Chapter 11: Primary Culture (1999). Such methods are not, however, directed to the isolation of nucleic acids. Instead, they are directed to degrading the structure of a tissue to allow cells to be isolated and cultured, a very different goal unrelated to nucleic acid isolation. Consequently, such methods are inoperable to achieve the embodiments of the invention because the methods are directed to optimizing cell viability, do not thoroughly break the bonds in the tissue, do not homogenize the tissue, and conventionally use different temperatures, concentrations, and/or durations of proteolytic exposure.

MEMS are conventionally useful only with cellular samples e.g., blood cells and microorganisms. A further advantage of certain embodiments of the invention, however, is that micromechanical devices may now be adapted to use with solid tissues using the present invention. Table 1 refers to conventional mechanical homogenization methods. A review of these methods shows that they use processes that are difficult to automate or adapt to a micromechanical deice. For example, sonication of tissue tends to cause heating and foaming. And grinders and glass beads are difficult to reduce in size. Although many μ-fluidic modules that have been demonstrated in the past decade to perform basic nucleic acid extraction and purification process, the sample preparation step is conventionally left off chip.

The reason is that the sample preparation process, unlike the nucleic acid isolation step, is varied and needs to be customized to the biological sample material (Huang et al., *Anal Bioanal. Chem*, 372:49-65, 2002).

TABLE 1: CONVENTIONAL MECHANICAL TISSUE HOMOGENIZATION

Cell disruption method	Application	General procedure
Sonication Ultrasonic waves generated by a sonicator lyse cells through shear forces. Complete shearing is obtained when maximal agitation is achieved, but care must be taken to minimize heating and foaming.	Cell suspensions	Sonicate cell suspension in short bursts to avoid heating. Cool on ice between bursts.
French pressure cell Cells are lysed by shear forces resulting from forcing cell suspension through a small orifice under high pressure.	Microorganisms with cell walls (bacteria, algae, yeasts)	Place cell suspension in chilled French pressure cell. Apply pressure and collect extruded lysate.
Grinding Some cell types can be opened by hand grinding with a mortar and pestle.	Solid tissues, microorganisms	Tissue or cells are normally frozen with liquid nitrogen and ground to a fine powder. Alumina or sand may aid grinding.
Mechanical homogenization Many different devices can be used to mechanically homogenize tissues. Handheld devices such as Dounce or Potter-Elvehjem homogenizers can be used to disrupt cell suspensions or relatively soft tissues. Blenders or other motorized devices can be used for larger samples. Homogenization is rapid and poses little danger to proteins except by the proteases that may be liberated upon disruption.	Solid tissues	Chop tissue into small pieces if necessary. Add chilled homogenization buffer (3–5 volumes to volume of tissue). Homogenize briefly. Clarify lysate by filtration and/or centrifugation.
Glass bead homogenization The abrasive actions of the vortexed beads break cell walls, liberating the cellular contents.	Cell suspensions, microorganisms	Suspend cells in an equal volume of chilled lysis solution and place into a sturdy tube. Add 1–3 grams of chilled glass beads per gram of wet cells. Vortex 1 minute and incubate cells on ice 1 minute. Repeat vortexing and chilling two to four times.

5

Indeed, different types of tissue samples require different treatments before nucleic acids can be extracted. The need for various treatments is a result of the inherent differences in the extracellular matrix compositions and inter-cellular connections in different tissues. For instance, muscle tissues and many cancer tissues are more fibrous and tougher in nature compared to brain or kidney tissue. These differences have lead to the conventional method of mechanically disrupting and homogenizing solid tissue by manually using an electric hand-held device, typically a Dounce or Potter-Elvehjem "homogeniser".

Despite increasing research on the automation for sample preparation in MEMs, much of the work has centred primarily on integrating simple cell lysis processes only. While many existing publications (e.g., U.S. Patent No. 6,344,326) have presented integrated approaches for DNA separation starting from cells, integrated microfluidic, MEM systems for nucleic acid isolation from solid tissue remain elusive and undemonstrated for two reasons: Firstly, cell samples are much easier to lyse and homogenise compared to tissue samples due to inter-cellular adhesions. Secondly, many standard methods for tissue homogenisation involve mechanical crushing and shearing forces, which are not MEMs friendly and pose significant obstacles to miniaturisation.

Such conventional manual and mechanical approaches to nucleic acid extraction approaches have been standard bench top processes for many years. Multitudes of nucleic acid isolation kits are available commercially. Many are non-automated (e.g. Ambion, Amersham, Qiagen, TRIzol kits, etc), providing only the chemical reagents and materials required for the nucleic acid isolation process. Some protocols like those of Dynal beads, incorporate automation into their isolation systems. However, these are at best semi-automated and still require a technician to perform many manual procedures and oversee the

process. For instance, in many "automated" nucleic acid isolation kits, the homogenization process for the preparation of cell lysate from tissue is still performed manually with an electric homogeniser, one sample at a time, resulting in the need for frequent washes of the homogeniser tip to prevent cross contaminations.

Certain embodiments of the invention, therefore, greatly simplify and improve the tissue disruption and homogenisation process. It helps to overcome many obstacles in bioMEMs in the process of sample preparation, and enable accelerated development of complete μ-TAS, which are capable of performing nucleic acid isolation from solid tissue in a completely automated fashion. An embodiment of the invention is a method wherein a clinician deposits a clinical sample in a receptacle and the entire nucleic acid isolation process takes place without further human intervention. The purified nucleic acid is collected in the chip and stored appropriately until required for further use.

A method of performing an embodiment of the invention is to perform a non-mechanical tissue homogenization step that may be followed by a cell disruption step. The tissue homogenization step includes exposing a tissue sample to a protease. The protease and tissue are preferably incubated in solution at a controlled temperature, preferably 37°C until tissue disruption visually appears to be complete. The tissue disruption is preferably performed so that the cells in the tissue remain intact. The cells may optionally be separated from the rest of the tissue, for example by a mechanical filtration step. The cells may optionally be sorted before lysis, for example by using an cell sorter that recognizes markers on the cells. The homogenized tissue product is optionally washed to remove proteases and is subjected to a cell disruption step, preferably performed by introducing the product into a lysis solution.

Conventional cell lysis techniques may be used to disrupt the intact cells. Table 2 describes some of these methods. Some of these methods may be used to preferentially recover one particular subcellular fraction. For example, conditions can be chosen in which only cytoplasmic fractions are released, or intact mitochondria or other organelles are recovered by differential centrifugation. Sometimes these techniques are combined, (e.g., osmotic lysis following enzymatic treatment, freeze-thaw in the presence of detergent). Proteases may be liberated when cells are lysed so that cell disruption is preferably performed at low temperatures. The sample may optionally be protected from proteolysis, and is preferable if the time between disruption and denaturation of cellular proteins is significant.

TABLE 2 CONVENTIONAL CELL LYSIS PROCESSES

Cell disruption method	Application	General procedure
Osmotic lysis Gentle method is well suited for applications in which the lysate is to be subsequently fractionated into subcellular components.	Blood cells, tissue culture cells	Suspend cells in a hypoosmotic solution.
Freeze-thaw lysis Many types of cells can be lysed by subjecting them to one or more cycles of quick freezing and subsequent thawing.	Bacterial cells, tissue culture cells	Rapidly freeze cell suspension using liquid nitrogen, then thaw. Repeat if necessary.
Detergent lysis Detergents solubilize cellular membranes, lysing cells and liberating their contents.	Tissue culture cells	Suspend cells in lysis solution containing detergent. Cells can often be lysed directly into sample solution or rehydration solution because these solutions always contain detergent.
Enzymatic lysis Cells with cell walls can be lysed gently following enzymatic removal of the cell wall. This must be done with an enzyme specific for the type of cell to be lysed (e.g., lysozyme for bacterial cells, cellulase and pectinase for plant cells, lyticase for yeast cells).	Plant tissue, bacterial cells, fungal cells	Treat cells with enzyme in isoosmotic solution.

The size of the tissue sample is preferably less than 100 µl, more preferably less than 50 µl, yet more preferably less than 10 µl, and even more preferably less than about 5 µl in volume. The smaller sizes are preferable so that penetration of the sample by a protease is facilitated. Tissue samples may be prepared, for example, by taking a biopsy of tissue with an appropriately sized biopsy tool. Or a tissue may be cut into tissue samples to achieve the desired volume. Embodiments of the invention are suitable for use with preserved tissues, including frozen tissues and tissues treated with preservatives, for example the product RNAlater.

Tissues may be plant, non-human animal, bacterial, and human tissues. Tissues include, without limitation: forensic, medical, agricultural, and research samples; tissues taken from live or dead sources; tissues processed immediately or stored until analysis; frozen, unfrozen, thawed, and never frozen tissues. The term tissue, as used herein, is an article that can be degraded by a protease or an enzymatic process. The tissues preferably contain at least two cells and a biomatrix. Extracellular matrices, polysaccharide matrices, and collagen are examples of a biomatrix.

The most preferred protease is collagenase since it degrades collagen, which is a chief component of most tissues. Other proteases, however, are suitable, including: papain, elastase, hyaluronidase, pronase, dispase, bromelain, cathespines, pepsin, and trypsin. Combinations of proteases may also be used. Some proteases are very specific in action and produce a limited cleaving action while others completely reduce a protein to individual amino acids. Accordingly, some proteases may be chosen if a particular tissue is known to be rich in a certain protein or biomolecule.

Certain embodiments of the invention include articles, preferably MEMS or bioMEMS, that include a proteolytic tissue homogenization chamber. A proteolytic tissue homogenization chamber refers to a chamber that accepts a tissue sample and an enzyme but does not accept or use a device for mechanically homogenizing tissue. Thus a proteolytic tissue homogenization chamber does not function with a mechanically acting device that homogenizes tissue, for example a grinder. And proteolytic tissue homogenization chamber does function to homogenize tissue by accepting a tissue sample and an enzyme, preferably one of the proteases disclosed herein, or an equivalent thereof. The proteolytic tissue homogenization chamber is preferably usable as a micromechanical device, and therefore is preferably adapted to use with small tissue samples and small volumes of enzymes. The chamber is preferably less than 100 μl in volume and the sample is preferably less than 100 μl in volume. Smaller volumes are more preferable, with a less than 50 μl volume being more preferable, a less than 10 μl volume being yet more preferable, and a less than 5 μl volume being most preferable.

The proteolytic tissue homogenization chamber preferably is operably associated with other chambers. The other chambers have other functions involved in tissue homogenization, cell disruption, or nucleic acid processing or analysis. Other chambers may include, without limitation: chambers for proteases, protease inhibitors, buffers, washes, detergents, chemicals, solutions, salts, or reagents; waste collection points; inlet ports; outlet ports; product collection chambers; and analysis chambers.

Separations processes may also be operably associated with the chambers described herein. For example, a filter may be used to separate homogenization products by size. Or other separations processes may be performed.

Inlets and outlets may be operably associated with the chambers described herein. For example, a chamber may have a LUER fitting for accepting an inlet stream and an outlet for directing the inlet stream to another chamber. For example, a source of proteases may be connected to a chip and the proteases pumped into a chip via a chamber.

Certain embodiments of the invention are a MEMS or bioMEMS device that incorporates on-chip sample preparation, including tissue homogenization using enzymatic methods as described herein. The MEMS may be single monolithic devices or several microfluidic modules, which are associated with or integrable with each other. The bioMEMS device may include processes of PCR amplification, electrophoresis, expression profile microarray analysis, genotyping, etc. Alternatively, the MEMS can be incorporated into an integrated micro-analytical system to perform the downstream amplification and detection functions after nucleic acid isolation which can be applied to diagnostics, drug discovery or biomedical research. Examples of MEMS or bioMEMS that perform some of these functions are found in U.S. Patent Nos. 6,675,817; 6,468,800; 6,468,761; 6,447,661; 6,440,725; 6,387,710; 6,375,817; 6,238,922; 6,221,677; 6,179,595; 5,952,215; 5,786,207; 5,667,985; 5,443,791; 5,374,395, which are hereby incorporated by reference herein.

EXAMPLES

Trypsins and collagenases were used as exemplary models of certain embodiments of the invention. The process set forth herein are applicable to other types of tissues, including human tissues.

Trypsin-EDTA digestion of rat liver was carried out as follows: freshly harvested tissue was cut into 2 mm^3 sample sizes, followed by washing twice in 500 µl iced Phosphate

Buffered Saline (PBS). Trypsin-EDTA solution was added to the tissue sample, which was incubated in a shaking water bath at 37°C for 30 min, and triturated from time to time until no further tissue disruption was observed. A similar procedure was followed using collagenase, except that: 1) incubation time was increased to 90 min and shaking was not necessary; and 2) gentle flicking of the sample was applied instead of trituration after incubation. The cell suspension obtained using these procedures yielded a homogenous solution that could be used for downstream RNA isolation by TRIzol directly without pelleting or washing the cells.

A series of experimental parameters were studied, including sample treatment, enzyme selection, enzyme concentration and volume, digestion duration and application of physical agitation. Cell viability counting was carried out as a direct monitoring of the digestion performance. RNA isolation from the cell suspension by TRIzol was conducted to examine the influence of enzymatic digestion in RNA preservation. RNA yield and purity were checked by UV-visible spectroscopy. RNA integrity was checked by agarose gel electrophoresis.

For sample treatment, incubation of sample in trypsin-EDTA at 4°C overnight before digestion was found to be comparable to the other methods used for tissue dissociation. It was also found that 2 mm^3 size of tissue, which is approximately the size of biopsy sample, was be effectively digested. Further dissection makes no significant difference in the digestion performance. As for enzyme selection, trypsin-EDTA, collagenase type I, IV and VIII were proven all effective in isolating cells.

As for enzyme concentration and volume, 0.01 % to 0.25 % trypsin-EDTA was effective, while 0.01 % to 0.15 % were found to be preferable. Other concentrations could be used, however, by adjusting the time of exposure to the protease. Generally, a higher enzyme

volume in a range of 20 µl to 500 µl afforded higher cell yields. Cell yield using 20 µl of trypsin enzyme was about 40 % of the yield from using 500 µl enzyme. For collagenase, 500 µl of 200 U/ml enzyme solutions were used for tissue digestion. As for digestion time, for trypsin-EDTA digestion, 30 min was found to be effective. For collagenase digestion, 1 to 2 hours was effective. Table 3 shows further experimental conditions.

Table 3: Experimental settings of tissue digestion by enzyme.

Enzyme type	Volume	Concentration	Reaction time	Agitation
Trypsin-EDTA	500 µl	0.05%	30 min	Shaking, pipetting
Collagenase type I	500 µl	200 U/ml	90 min	Flicking
Collagenase type IV	500 µl	200 U/ml	90 min	Flicking
Collagenase type VIII	500 µl	200 U/ml	90 min	Flicking

Cell viability counting results showed that the number of cells isolated from 10 mg (2 mm^3) rat liver tissue varied between 2.0×10^6 to 5.0×10^6. Cell viability evaluated by trypan blue was found to be between 97 % to 100 %.

RNA isolated from enzyme digestion approach was compared with that from a conventional homogenization approach. Gel electrophoresis images of total RNA are shown in Figure 2: Agarose gel of total RNA run in TBE. Lane from left to right: Lane 1: high range RNA marker 6 kb, 4 kb, 3 kb, 2 kb, 1.5 kb, 1 kb, 0.5 kb; Lane 2: low range RNA marker 1 kb, 0.8 kb, 0.6 kb, 0.4 kb, 0.3 kb; Lane 3: total RNA isolated by collagenase type I; Lane 4: total RNA isolated by collagenase type IV; Lane 5: total RNA isolated by collagenase type VIII; Lane 6: total RNA isolated by trypsin-EDTA; Lane 7: total RNA isolated by homogenization. The presence of the two distinctive rRNA bands at 28 S and 18 S indicates the total RNA species were well-preserved.

In general, the approach afforded similar results to conventional processes. The RNA yield was 50 – 100 mg from 10 mg rat liver tissue, which was comparable to that reported

elsewhere using homogenization (60 –100 mg; Invitrogen Protocol). An OD ratio of A260 to A280 was found to be 2.08 to 2.12 measured in PH 7.4 PBS buffer, which indicates the RNA was of high purity.

One possible scheme for implementing enzymatic tissue digestion in a bioMEM system is shown in Figure 3, which depicts the design of a μ-fluidic cartridge consisting of (1) chambers for buffer and protease solutions; (2) inlet and reaction ports for a solid tissue sample; (3) a collection port for the digested solution; and (4) a waste chamber. In addition, the illustrated μ-fluidic cartridge could also be integrated with other downstream bioMEMs processes, such as cell lysing, nucleic acid separation and detection.

Alternative embodiments of the invention are shown in Figure 4, which depicts another two simplified variations of the μ-fluidic cartridge. Figure 4A and 4B are single layer and double layer m-tissue digesters (μ-TD) respectively. Figure 4C is a photographic image of the single layer μ-TD made in PDMS. The μ-TD can be made in a wide range of materials typically used for microfabricated systems. These include but are not limited to materials such as a silicon wafer, silica wafer, polydimethylol siloxane (PDMS), polycarbonate and polymethyl methacrylate (PMMA).

The operation of the μ-TD of Figure 4 and the usage of the interconnections as sown in Figure 5 are described as follows. The tissue sample is first inserted into the middle "tissue inlet and reaction port" of the μ-TD. The μ-TD that contains the tissue sample is subsequently placed on top of the bottom piece (Y) of the interconnection (Fig. 5) and then covered by the top piece (X) of the interconnection. The top piece (X) of the interconnection is designed to seal the "tissue inlet and reaction port", leaving only the reagents inlet and sample collection reservoir connected to the openings (Z1 & Z2) in the top piece (X) of the

interconnection. Reagents will be inserted into the m-TD via these openings in the top piece (X) of the interconnection. Tubing is connected to openings (Z1 & Z2) on the top piece of interconnection and pressure-driven flow is controlled by a syringe pump.

This particular example illustrated a design of μ-TD that used an enzymatic tissue digestion method as described in this invention and showed interconnections used for the purposes of operating the μ-TD. Other μ-TD designs and interconnections can also be used.

* * *

The patents, patent applications, and publications set forth in this application (including the appendices of the application) are hereby incorporated by reference herein. The embodiments of the invention set forth herein are merely exemplary and are not intended to limit the scope of the invention.

CLAIMS

1. The inventions as described herein.

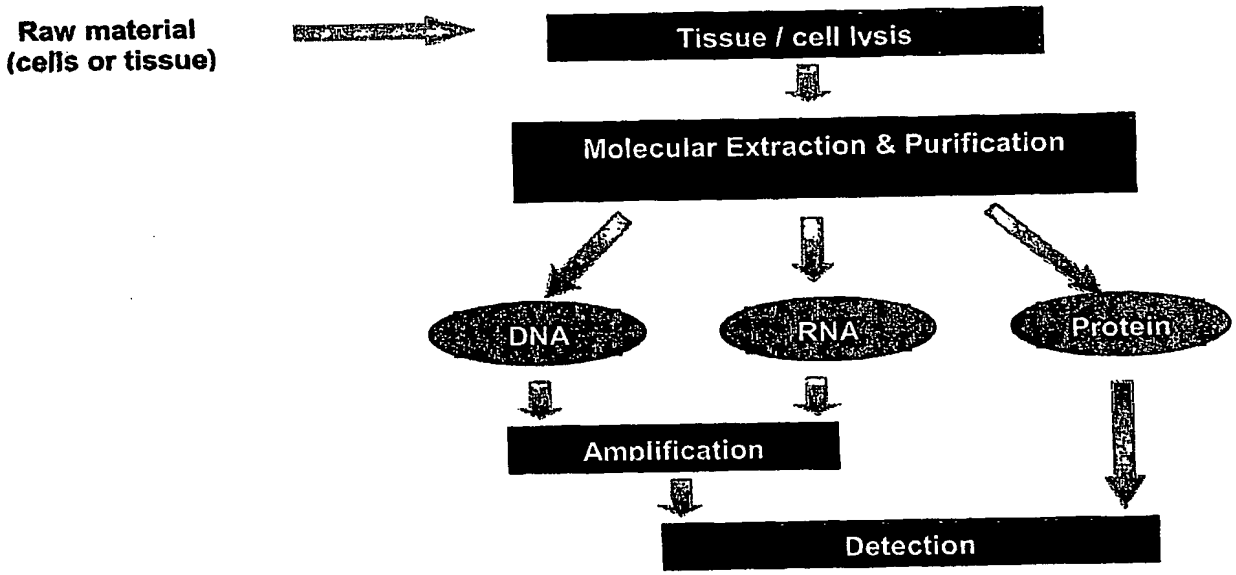
Raw material
(cells or tissue)
Tissue / cell lysis
Molecular Extraction & Purification
DNA
RNA
Protein
Amplification
Detection

FIGURE 1

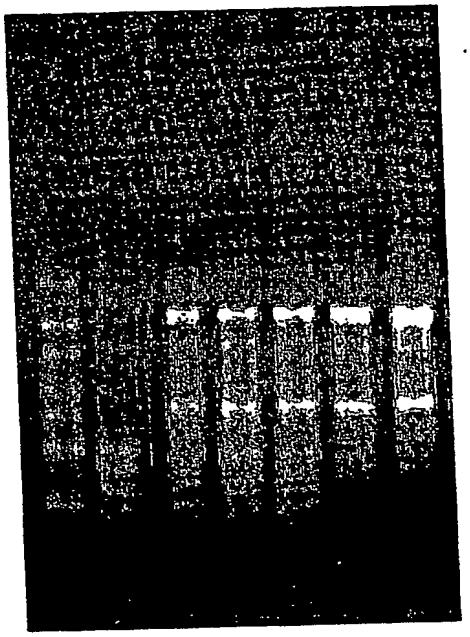

Figure 2: lane 1 is at left.

60427148 . 111802

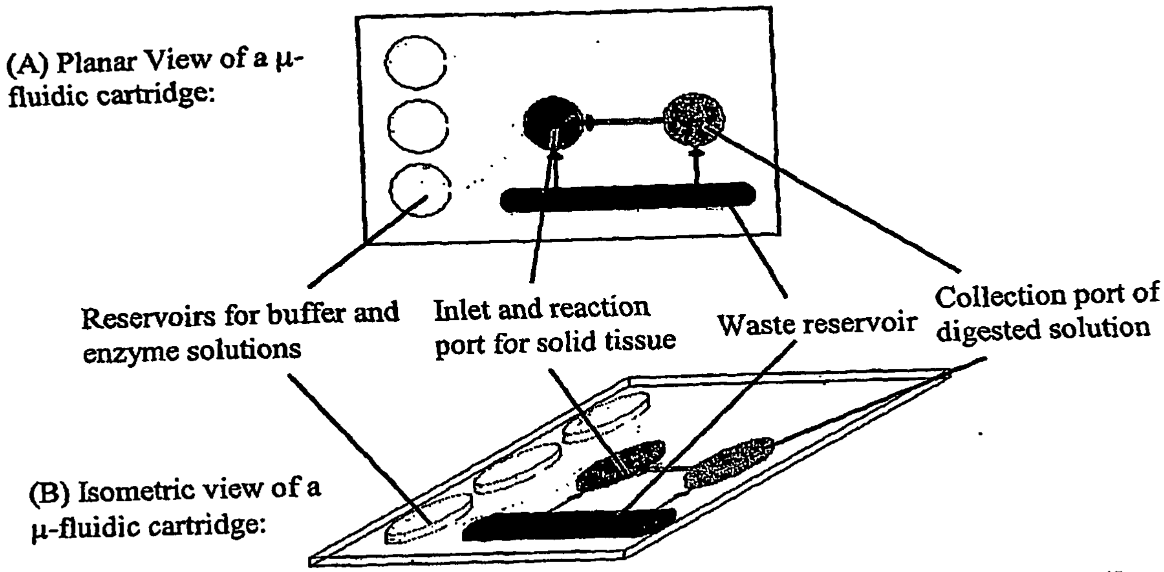

Figure 3. Design of a μ-fluidic cartridge utilising the enzymatic tissue digestion system (for illustrative purpose).

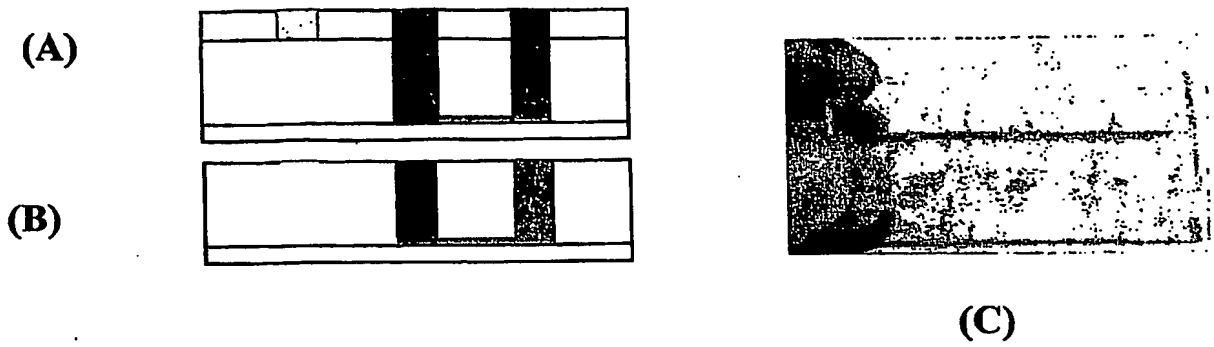

FIGURE 4

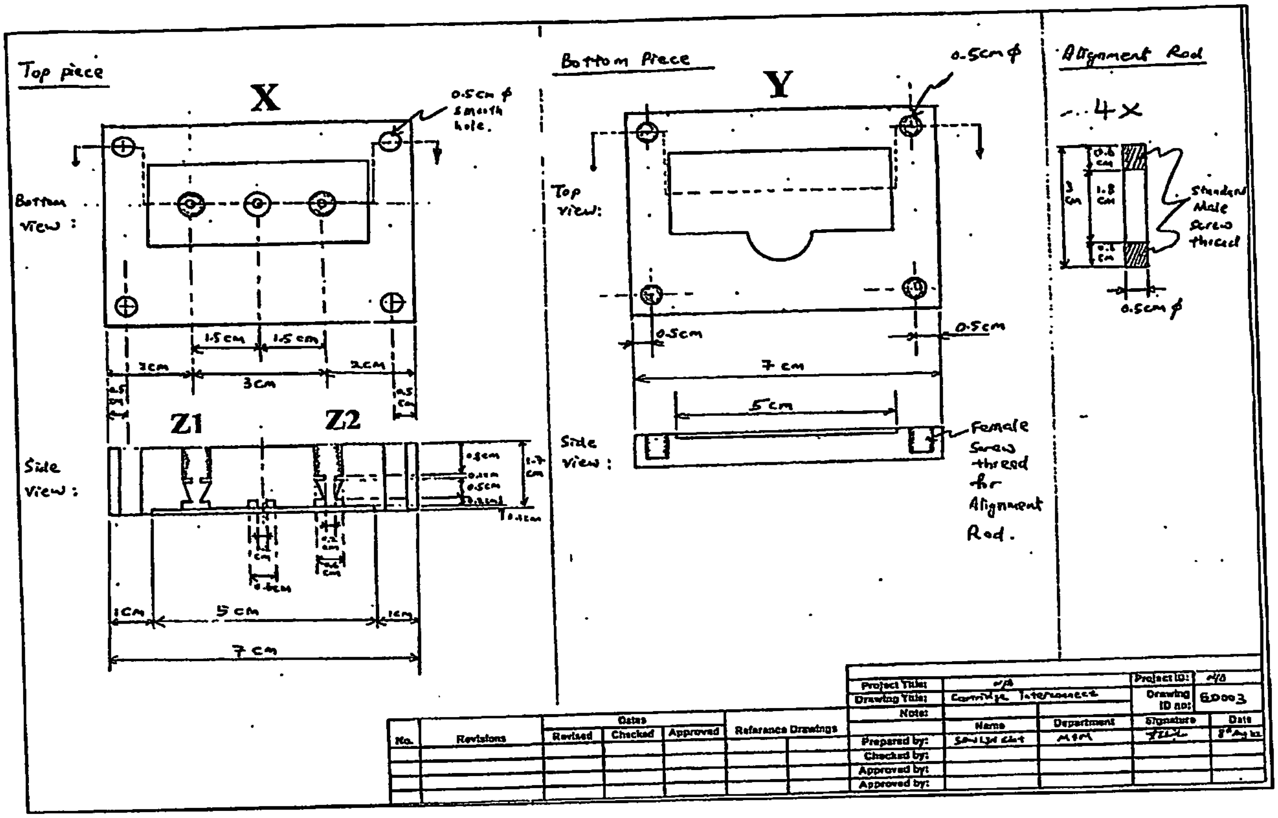
Top piece
X
0.5cm ϕ smooth hole.
Bottom view :
1.5cm
1.5cm
2cm
3cm
2cm
Z1
Z2
Side view :
1cm
5cm
1cm
7cm
Bottom Piece
Y
0.5cm ϕ
Top view:
0.5cm
0.5cm
7cm
5cm
Side view :
Female Screw thread for Alignment Rod.
Alignment Rod
4X
Standard Male Screw thread
0.5cm ϕ
Project Title:
Drawing Title:
Note:
Project ID:
Drawing ID no:
60003
Name
Department
Signature
Date
Prepared by:
Checked by:
Approved by:
Approved by:
No.
Revisions
Dates
Revised
Checked
Approved
Reference Drawings

FIGURE 5